

TCOF1 in extracellular vesicles predicts survival in patients with HCC treated with high-dose conformal radiotherapy

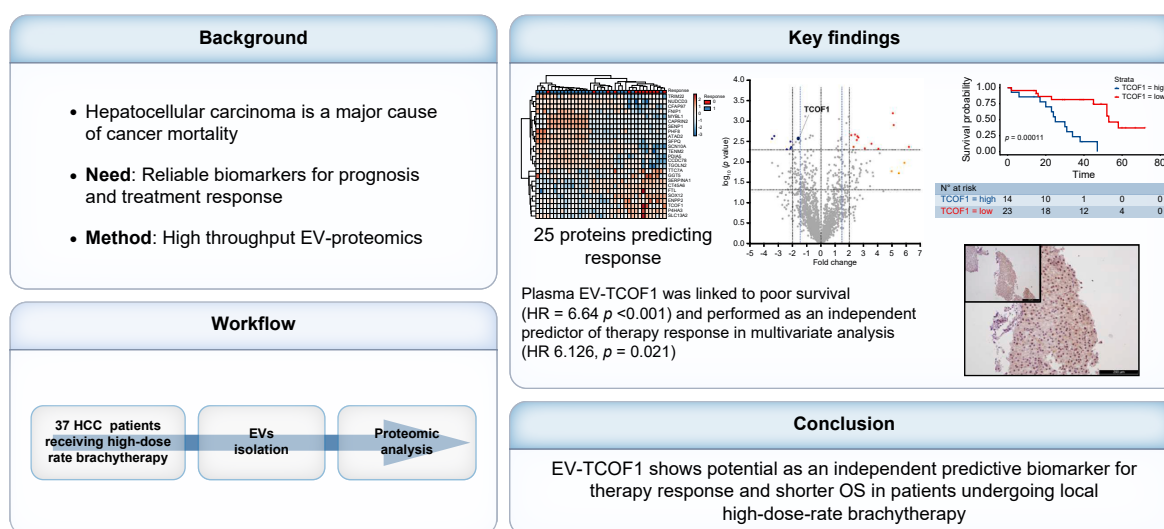
Authors

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Graphical abstract



Highlights:

- HCC lacks reliable minimally invasive biomarkers, limiting accurate prediction of treatment response and survival.
- Extracellular vesicle proteomic profiling identified distinct protein signatures associated with response to high-dose-rate brachytherapy.
- EV-TCOF1 emerged as an independent prognostic biomarker linked to poorer overall survival.
- These findings highlight the clinical potential of EV-based liquid biopsy approaches for improved risk stratification.

Impact and implications:

This study addresses the need for reliable, minimally invasive biomarkers to predict treatment response and survival in patients with HCC undergoing radiation therapy. As a component of liquid biopsy, circulating extracellular vesicles provide a non-invasive window into tumor biology. EV-associated TCOF1 may inform risk-adapted treatment planning, surveillance strategies, and the identification of patients who could benefit from alternative therapies. More broadly, these findings support the integration of EV-based biomarkers into translational research and the development of non-invasive approaches to personalized cancer care.

TCOF1 in extracellular vesicles predicts survival in patients with HCC treated with high-dose conformal radiotherapy

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Background & Aims: Hepatocellular carcinoma (HCC) is a leading cause of cancer-related mortality worldwide. The lack of reliable biomarkers to predict treatment response limits patient stratification and personalized therapy. Extracellular vesicles (EVs) represent a minimally invasive source of tumor-derived proteins that may reflect treatment-relevant biological changes. This study investigated EV-based protein profiling in patients with HCC undergoing high-dose conformal radiotherapy to identify prognostic biomarkers associated with treatment response and overall survival.

Methods: Plasma samples from 37 patients with HCC treated with high-dose-rate interstitial brachytherapy (HDR-BT) were analyzed. EVs were isolated using EVTrap magnetic beads (Tymora Analytical, West Lafayette, IN, USA). Proteomic profiling was performed by mass spectrometry to identify differential protein expression between responders and non-responders. Statistical analyses included Fisher's exact and Mann-Whitney *U* tests. Survival was assessed using Kaplan-Meier and log-rank tests, and prognostic factors were evaluated by univariate and multivariate Cox regression. A two-sided *p* < 0.05 was considered significant.

Results: A total of 2,187 unique EV proteins were identified, of which 25 showed differential expression between patients' subgroups. EV-TCOF1 (treacle ribosome biogenesis factor 1) was strongly associated with poor survival, with a hazard ratio of 6.64 (CI 2.24–19.69) and *p* < 0.001. Multivariate analysis confirmed EV-TCOF1 as an independent predictor of overall survival. Immunohistochemistry analysis supported the tumoral origin of EV-TCOF1. External validation further demonstrated TCOF1 as a robust prognostic marker.

Conclusions: EV-TCOF1 emerged as a candidate biomarker associated with therapy response and shorter overall survival in an explorative cohort of patients with HCC undergoing local high-dose conformal radiotherapy. These findings highlight the potential of EV-based proteomics as a minimally invasive tool to refine prognostic assessment in HCC and the emerging role of EV-TCOF1 specifically warrants further investigation to validate its clinical utility.

Clinical Trials registration: German Clinical Trials Register DRKS00010587.

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver malignancy and represents a major global health burden. According to the 2020 GLOBOCAN data, HCC accounts for over 900,000 new cases and over 800,000 deaths worldwide annually ranking among the leading causes of cancer-related mortality.¹ Despite advances in diagnostic imaging and therapeutic approaches, the prognosis of HCC remains poor because of late diagnosis and frequent tumor recurrence. Currently, the clinical management of HCC is challenging owing to the lack of reliable, minimally invasive biomarkers that can accurately predict disease progression and therapeutic response. Alpha-fetoprotein (AFP), the most commonly used serum biomarker, has limited sensitivity and specificity, necessitating the development of novel biomarkers for

improved patient stratification and personalized treatment.² With a growing variety of treatment options across different disease stages including local, locoregional, and systemic therapies and their combinations, biomarkers gain particular attention and importance for therapy guidance.^{3,4} Among locoregional treatment, high-dose-rate interstitial brachytherapy (HDR-BT) represents a form of high-dose conformal radiotherapy in which radiation is delivered directly to the tumor via intratumoral or peritumoral applicators, resulting in highly localized dose distributions with steep dose gradients. This approach is conceptually distinct from stereotactic body radiotherapy (SBRT), which relies on external beam delivery of high-dose radiation in a fractionated manner.

Extracellular vesicles (EVs) are now recognized as key mediators of a wide range of biological processes, including

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intercellular communication, immune modulation, and disease progression.^{5–7} Their biological activity is determined by the diverse cargo of proteins, nucleic acids, and lipids encapsulated within their lipid bilayers, which reflect the physiological or pathological state of the cells from which they originate. In cancer, tumor-derived EVs can disseminate oncogenic signals both locally and systemically, thereby fostering tumor growth, immune escape, and resistance to therapy.⁸ Proteomic profiling of EVs has revealed context-dependent alterations in protein composition, indicating that EV cargo dynamically mirrors disease stage and therapeutic response.⁹ Accordingly, EVs have been actively explored as a minimally invasive source of predictive and prognostic biomarkers, with broad potential applications in precision oncology.¹⁰ In line with this, our group has reported similar observations in an independent study, further underscoring the robustness and translational relevance of EV-based proteomics as predictive biomarkers in oncology.¹¹ The aim of this study was to characterize EVs protein profiles in patients with HCC undergoing HDR-BT and to identify potential prognostic biomarkers associated with treatment response and overall survival.

Patients and methods

Ethical considerations

This study represents a sub-analysis of patients with HCC treated with CT-guided HDR-BT at the Department of Radiology, LMU University Hospital. All study protocols were approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki (German Clinical Trials Register-ID: DRKS00010587). Before study enrollment, all patients were fully informed by the treating physician of the scope and goals of the study, had given written informed consent, and were willing to comply with the study protocol.

Study population and design

The study population represents a subcohort of the ESTIMATE study patients and consists of 37 patients with HCC undergoing local tumor ablation with HDR-BT. Details of subject selection can be obtained from Fig. 1S. Patients were recruited between August 2017 and December 2023. Written informed consent was obtained for the local ablative treatment as well as the study inclusion. Median follow-up was 38 months. Patients with HCC Barcelona-Clinic Liver Cancer (BCLC) A and B were eligible if not treated previously and no immunosuppressive therapy or immunosuppression present, as previously reported.¹²

Treatment and response assessment

Stratification of patients into responders and non-responders was conducted as previously described,¹² based on the following clinical and imaging criteria: responders had neither limited (up to BCLC A), nor diffuse or systemic tumor progression within 6 months of the index procedure and at most localized progression from 6 months to 2 years not exceeding three nodules/nodule diameter >3 cm. Non-responders had HCC recurrence within 6 months and/or tumor progression with more than three nodules and/or nodule size exceeding 3 cm and/or extrahepatic disease within 2 years of the index procedure. Two patients underwent liver transplantation 8 and

10 months after tumor ablation and were classified as responders without progression until transplantation and with no evidence of viable tumor in explanted livers. Representative magnetic resonance imaging (MRI) images from responders and non-responders are presented in Fig. 2S.

Sample collection

Peripheral venous blood (5 ml) was collected at baseline and at follow-up (6–9 weeks from therapy) in Monovette EDTA tubes (Sarstedt AG, Nümbrecht, Germany) and processed immediately (centrifugation 1,300 g, 5 min, 4 °C). Plasma aliquots were stored at -80 °C until use. Tissue samples were obtained through an 18-gauge coaxial biopsy needle, fixed in 10% formalin overnight at 4 °C, and subsequently embedded in paraffin.

EV isolation, characterization, and mass spectrometry analysis

In total, 37 plasma samples (100 µl) were analyzed by Tymora Analytical (West Lafayette, IN, USA) and used for enrichment of EVs using EVTrap magnetic beads as previously described.^{11,13,14} EV extraction was validated by tunable resistive pulse sensing (TRPS) with 150 nm nanopores for size distribution and by Western blot analysis.¹⁵ In total, 1 mg of peptides from each sample was subjected to mass spectrometry analysis. Raw data were generated and processed according to published methodology.¹⁶ The abundance levels of all peptides and proteins were normalized to the spiked-in internal indexed retention time standard.¹⁶

Differentially expressed protein and Gene Ontology enrichment analysis

Differentially expressed proteins (DEP) analysis was performed between responders and non-responders with the threshold of 1.5-fold change and false discovery rate (FDR)-adjusted *p* value <0.05, followed by hierarchical clustering. The lists of DEP were then subjected to BioVenn web-based Venn diagram analysis¹⁷ and ShinyGO (v0.65) Gene Ontology (GO)-enrichment analysis¹⁸ with enrichment FDR-adjusted *p* value <0.05 and gene sets of GO biological process, GO Cellular Component, and GO molecular function.

Immunohistochemistry staining

Tumor blocks collected before therapy were available for a limited number of patients only. Serial tissue sections (2 µm) were cut from formalin-fixed, paraffin-embedded tumor tissues, dewaxed, and rehydrated according to standard procedure (preheating at 60 °C; deparaffinization in Neo-Clear, Merck KGaA, Darmstadt, Germany; rehydration in graded series of ethanol and distilled water) and stained with H&E for determination of tissue morphology. For immunohistochemistry analysis, the primary antibody anti-TCOF1 (treacle ribosome biogenesis factor 1; rabbit polyclonal, catalog no. ab224544, dilution 1:100, Abcam, Cambridge, UK) was applied overnight at 4 °C, followed by incubation with the secondary antibody (goat Anti-Rabbit IgG [HRP]; catalog no. ab205718, dilution 1:200, Abcam). DAB+ chromogen (DAKO Liquid DAP+ Substrate Chromogen System, Agilent Technologies Inc., Santa Clara, CA, USA) was used as chromogen.

Sections were counterstained with hematoxylin, dehydrated, and mounted using Neo-Mount (Merck KGaA).

Statistical analysis

Normality of the variables was assessed using the Shapiro–Wilk test. For continuous parameters, Fisher’s exact test was used, whereas the Student’s *t*-test or the Mann–Whitney *U* test was applied for categorical parameters. GO enrichment analysis was performed using ShinyGO.¹⁸ Receiver operating characteristic (ROC) curve analysis identified optimal protein cut-off values that maximized sensitivity and specificity for predicting individual survival. Volcano plots display $\log_2$ fold changes versus $-\log_{10}$ adjusted *p*-values derived from differential expression analysis. Overall survival (OS) was estimated using the Kaplan–Meier method, and statistical significance was evaluated using the log-rank test. Optimal cut-off values were determined using the Youden index. Univariate and multivariate Cox proportional hazards regression analysis was conducted to identify prognostic factors. Variables with a *p* value <0.05 in the univariate analysis were included in the multivariate analysis. For all analyses, a two-sided *p* value of <0.05 was considered statistically significant. Given the limited sample size, univariate and multivariate analyses must be considered exploratory. Statistical analysis was performed with R statistical and computing software, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria), GraphPad Prism, version 10.2.3 (GraphPad, San Diego, CA, USA), and IBM SPSS, version 29.0 (IBM, Armonk, NY, USA).

Results

Baseline patients’ characteristics

A total of 37 patients with HCC (mean age 69.5, range 44–86) were included in this study and treated with HDR-BT. With respect to tumor stage, 34 patients were classified as BCLC A and three patients as BCLC B.¹⁹ Most patients had Child–Pugh A liver cirrhosis and underwent local ablation of one single lesion. Besides total bilirubin (*p* = 0.03), none of the other clinical parameters differed significantly between responders and non-responders. Baseline characteristics are summarized in Table 1 and Fig. 1A.

EVs isolation and characterization

EVs were extracted and isolated as described previously.¹¹ Examination by TRPS of the eluted post-EVTrap EVs demonstrated the expected diameter range between 100 and 200 nm (mean particle diameter: 123.0 nm, range 55.0–495.0 nm, SD:67.0). EV characterization by Western blot analysis revealed that EV marker proteins, CD9, and newly identified pan-EV marker, Moesin (MSN¹⁶), were present in the samples (Fig. 1B). The proteomes of the plasma EVs from all samples were characterized according to the ISEV2018 recommendations.²⁰ Contaminants such as ALB, APOA1/2, HSP90B1, and CANX were present evenly across different samples as background noise.

Proteomic profile of plasma EVs

To identify potential EV-associated biomarkers, we performed a comprehensive proteomic analysis, which revealed a total of

2,187 unique proteins. Comparison with the publicly available databases Vesiclepedia²¹ and ExoCarta,²² demonstrated that ~70% of the isolated EV proteins overlapped with the top 100 most frequently reported EV-associated proteins, confirming the robustness of the EV isolation and characterization (Fig. 1C). Supervised clustering with the whole proteome of the isolated EVs showed a distribution of the proteins according to the therapy response (Fig. 1D). The identities and subcellular localizations of the detected proteins were verified through GO enrichment analysis, which categorized them into GO Cellular Component, biological processes, and molecular functions (Fig. 1E). Within the GO Cellular Component, the proteins were significantly enriched in blood microparticles, extracellular vesicles, extracellular organelles, and exosomes (enrichment *p* <0.0001), confirming their EV origin. GO Biological Process analysis revealed predominant involvement in degranulation, exocytosis, and secretion, whereas GO molecular function analysis indicated enrichment in pathways related to cell adhesion and molecular binding.

Analysis of the differential protein abundance of EVs and association with survival risk

To determine the differential expression between the EV proteins isolated from responders and non-responders, an unpaired differential expression analysis was conducted on baseline plasma samples. According to relative abundance (fold change < -1.5 or > +1.5) and statistical significance (*p* <0.005), we identified 10 overexpressed unique EV proteins among non-responders and 15 overexpressed unique proteins among responders (Fig. 2A–C). Pearson correlation analysis revealed a higher number of significant correlations among proteins in responders compared with non-responders. Setting a threshold of *p* <0.01 and $|r| \geq 0.7$, in non-responders, a significant unique correlation was observed between SOX12 and ENPP2 (*r* = 0.70). Among responders, a strong correlation was detected between TENM2 and SFPQ (*r* = 0.77), CCDC78 and PDIA5 (*r* = 0.79), ATAD2 and SFPQ (*r* = 0.95), ATAD2 and TENM2 (*r* = 0.73), PHF8 and SFPQ (*r* = 0.72), SENP1 and ATAD2 (*r* = 0.70), TGOLN2 and CCDC78 (*r* = 0.88), SCN10 and CFAP97 (*r* = 0.75), MYBL1 and SENP1 (*r* = 0.97), CAPRIN2 and ATAD2 (*r* = 0.71), and CAPRIN2 and SENP1 (*r* = 0.97) (Fig. 2D). Functional enrichment analysis revealed that the predominant biological processes associated with the proteins identified in non-responders included response to lithium ions, redox metabolism, rRNA transcription and cell morphology regulation, large rRNA transcription in the nucleus, and regulation of lamellipodium morphogenesis, whereas for responders the proteins were mainly associated with transcriptional activation and chromatin organization (Fig. 2E).

Prognostic value of EV proteins assessed by survival and ROC analyses

To establish the clinical relevance of the identified proteins, we next assessed their association with patient OS and evaluated their predictive performance. Kaplan–Meier survival analyses revealed that patients with elevated levels of the 10 high-risk proteins experienced significantly shorter OS, whereas those with higher levels of the 15 protective proteins showed prolonged survival. These differences were statistically supported

Table 1. Baseline characteristics. Categorical variables were analyzed using Fisher's exact test, and continuous variables using the Mann-Whitney U test.

Variable	All N = 37	Responders n = 24	Non-responders n = 13	p value
Age				
Median (range)	69.5 (44–86)	69.6 (51–85)	69.2 (44–86)	0.98
Sex, n (%)				
Female	8 (21.6)	5 (20.8)	3 (23.1)	1.00
Male	29 (78.4)	19 (79.2)	10 (76.9)	
Treated lesions, n (%)				
Patients with one lesion	27 (72.9)	19 (79.2)	8 (61.5)	0.44
Patients with two lesions	10 (26.1)	5 (20.8)	5 (38.5)	
Catheters' number, n (%)				
1–3	33 (89.2)	22 (91.6)	11 (84.6)	0.60
4–8	4 (10.8)	2 (8.4)	2 (15.4)	
Lesion size (cm), (mean, range)	4.01 (1.1–10.9)	3.70 (1.70–6.90)	4.12 (1.1–10.9)	0.84
AFP (median, range)	5.20 (1.60–35,754)	5.20 (1.60–742.00)	5.25 (1.70–35,754)	0.75
CPS, n (%)				
A	28 (75.6)	19 (79.2)	9 (69.2)	0.69
B	9 (24.4)	5 (20.8)	4 (30.8)	
Cirrhosis, n (%)				
No	6 (16.2)	5 (20.8)	1 (7.7)	0.39
Yes	31 (83.8)	19 (79.2)	12 (92.7)	
MASH, n (%)				
No	32 (94.1)	21 (87.5)	11 (84.6)	0.39
Yes	5 (5.9)	3 (12.5)	2 (15.4)	
Etiology, n (%)				
Hepatitis	10 (27.0)	7 (29.1)	3 (23.1)	0.91
MASH/alcohol	15 (40.5)	9 (37.5)	6 (46.2)	
Cryptogenic	12 (32.5)	8 (33.4)	4 (30.7)	
Alcohol intake, n (%)				
No	22 (59.5)	14 (58.3)	8 (61.5)	0.44
Yes	14 (37.8)	10 (41.7)	4 (30.8)	
Former alcohol intake	1 (2.7)	0 (0.0)	1 (7.7)	
Chronic hepatitis B, n (%)				
No	33 (89.2)	23 (95.8)	10 (76.9)	0.11
Yes	4 (10.8)	1 (4.2)	3 (23.1)	
Chronic hepatitis C, n (%)				
No	30 (81.1)	18 (75)	12 (92.3)	0.37
Yes	3 (8.1)	2 (8.3)	1 (7.7)	
Inactive infection	4 (10.8)	4 (16.7)	0	
Total bilirubin (μmol/L) (mean, range)	1.21 (0.30–2.60)	1.16 (0.30–2.60)	1.43 (1.2–3.60)	0.03
Albumin (g/dl) (mean, range)	3.66 (2.60–4.70)	4.00 (2.60–4.70)	3.00 (2.60–4.50)	0.40
INR (mean, range)	1.13 (0.9–1.6)	1.11 (0.9–1.6)	1.16 (0.9–1.5)	0.16
Ascites, n (%)				
No	18 (48.6)	11 (45.8)	7 (53.8)	0.73
Yes	19 (51.4)	13 (54.2)	6 (46.2)	
Hepatic encephalopathy, n (%)				
No	34 (91.9)	23 (95.8)	11 (84.6)	0.54
Yes	3 (8.1)	1 (4.2)	2 (15.4)	
Liver volume (cm ³) (mean, range)	1,524.16 (823–2,975)	1,535.37 (823–2,975)	1,503.46 (868–2,159)	0.99
Liver >5 Gy (cm ³) (mean, range)	299.30 (69.3–1,036)	264.66 (73–729)	363.25 (69.3–1,036)	0.61
Liver 5 Gy/liver vol (%) (mean, range)	19.87 (4.67–52.19)	18.27 (4.67–50.76)	22.83 (5.76–52.19)	0.48
Clinical target volume, sum (cm ³) (mean, range)	48.11 (1.90–385)	34.83 (3.2–127)	74.67 (1.90–385)	0.71
10Ci time (s) (mean, range)	887.81 (280–3,204)	792.54 (338–1,847)	1,063.70 (280–3,204)	0.64

AFP, alpha-fetoprotein; CPS, Child–Pugh class; INR, international normalized ratio; MASH, metabolic dysfunction-associated steatohepatitis.

by the log-rank test, underscoring the prognostic value of these proteins (Table 2).

Cox proportional hazard analysis was performed for each of the 25 identified proteins (classified as high or low according to their respective mean value) to evaluate their association with patient survival outcomes. The higher risk of poor survival was observed in patients with elevated concentration of EV-TCOF1 (hazard ratio [HR] 6.642, $p < 0.001$), followed by EV-TTC7A (HR 3.425, $p = 0.009$), EV-SOX12 (HR 3.344, $p = 0.017$), EV-SLC13A2 (HR 3.16, $p = 0.012$), EV-ENPP2 (HR 3.057, $p = 0.014$), EV-P4H3 (HR 2.841, $p = 0.02$), and EV-GGT5 (HR 2.811, $p = 0.026$). Conversely,

patients exhibited the lowest risk of poor survival with higher concentrations of EV-CCDC78 (HR 0.188, $p = 0.001$), followed by EV-CAPRIN2 (HR 0.204, $p = 0.011$), EV-TGOLN2 (HR 0.222, $p = 0.0004$), EV-SCN10A (HR 0.242, $p = 0.018$), EV-PDIA5 (HR 0.283, $p = 0.008$), EV-SEN1 (HR 0.293, $p = 0.05$), EV-NUDCD3 (HR 0.296, $p = 0.016$), and EV-CFAP97 (HR 0.321, $p = 0.015$) (Fig. 3A). To further quantify the discriminative ability of the proteins, we performed ROC curve analysis. This independent evaluation identified six proteins with an area under the curve (AUC) exceeding 0.7, indicating moderate to good discriminative potential for survival outcomes (Fig. 3B and C). The full list of proteins

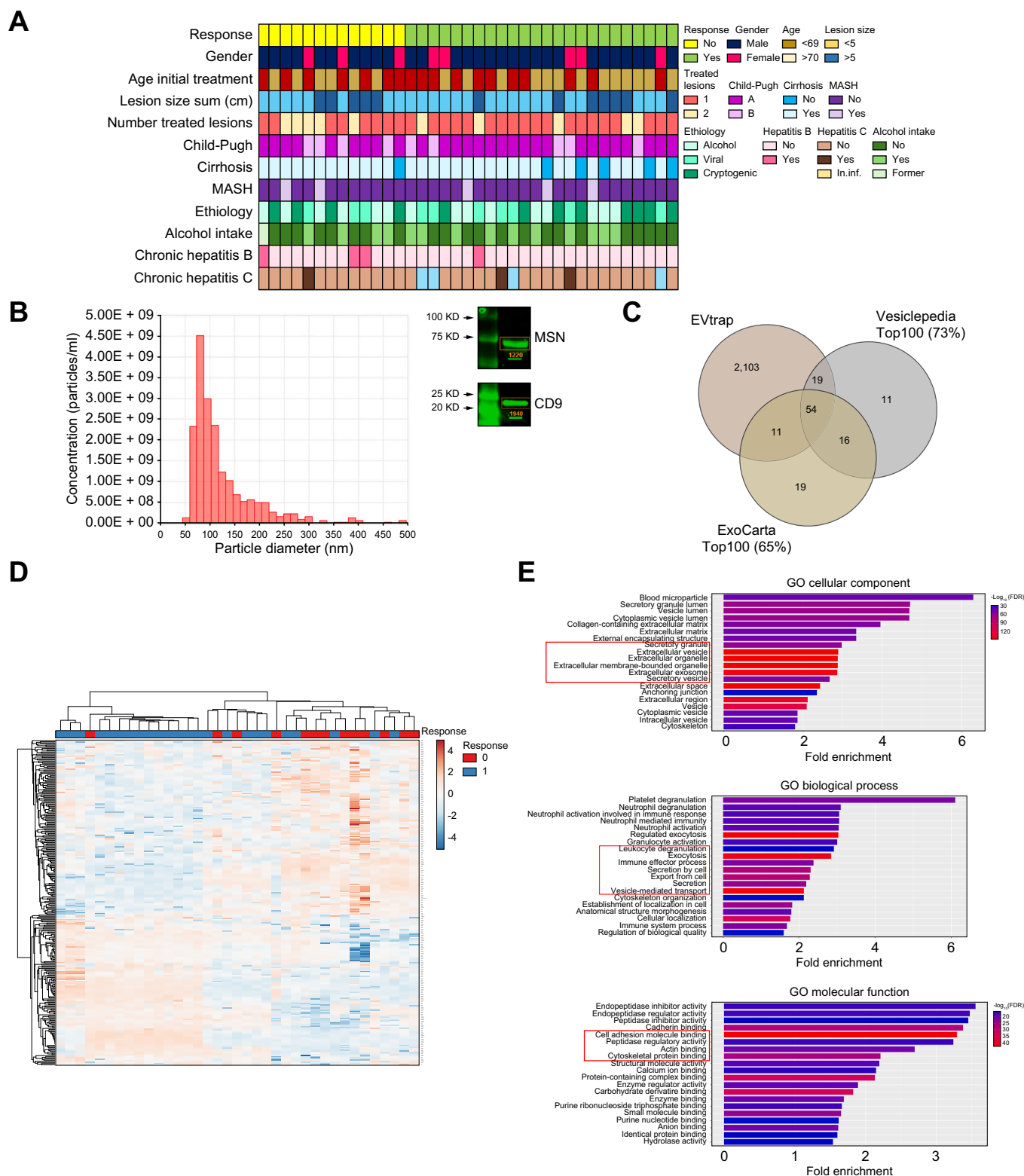


Fig. 1. Isolation and characterization of plasma EV isolated from patients with HCC. (A) Patients' characteristics. (B) Size distribution of a representative plasma EV determined by tunable resistive pulse sensing (TRPS) and Western blot validation of EV markers (MSN and CD9). (C) Venn diagram showing the overlap of proteins identified from HCC-derived plasma EVs with top 100 proteins published in the Vesiclepedia and ExoCarta databases. (D) Heatmap of the differential level of isolated proteins. Legend: 0 = NR, 1 = R. (E) Distribution of detected proteins across GO categories. GO Cellular Component analysis showed significant enrichment in extracellular vesicle-related terms, confirming the EV origin of the isolated proteins. GO biological process analysis revealed enrichment in pathways related to degranulation, exocytosis, and secretion, whereas GO molecular function analysis highlighted proteins involved in cell adhesion and molecular binding pathways. EV, extracellular vesicle; HCC, hepatocellular carcinoma; MSN, Moesin; GO, Gene Ontology; NR, non-responders; R, responders.

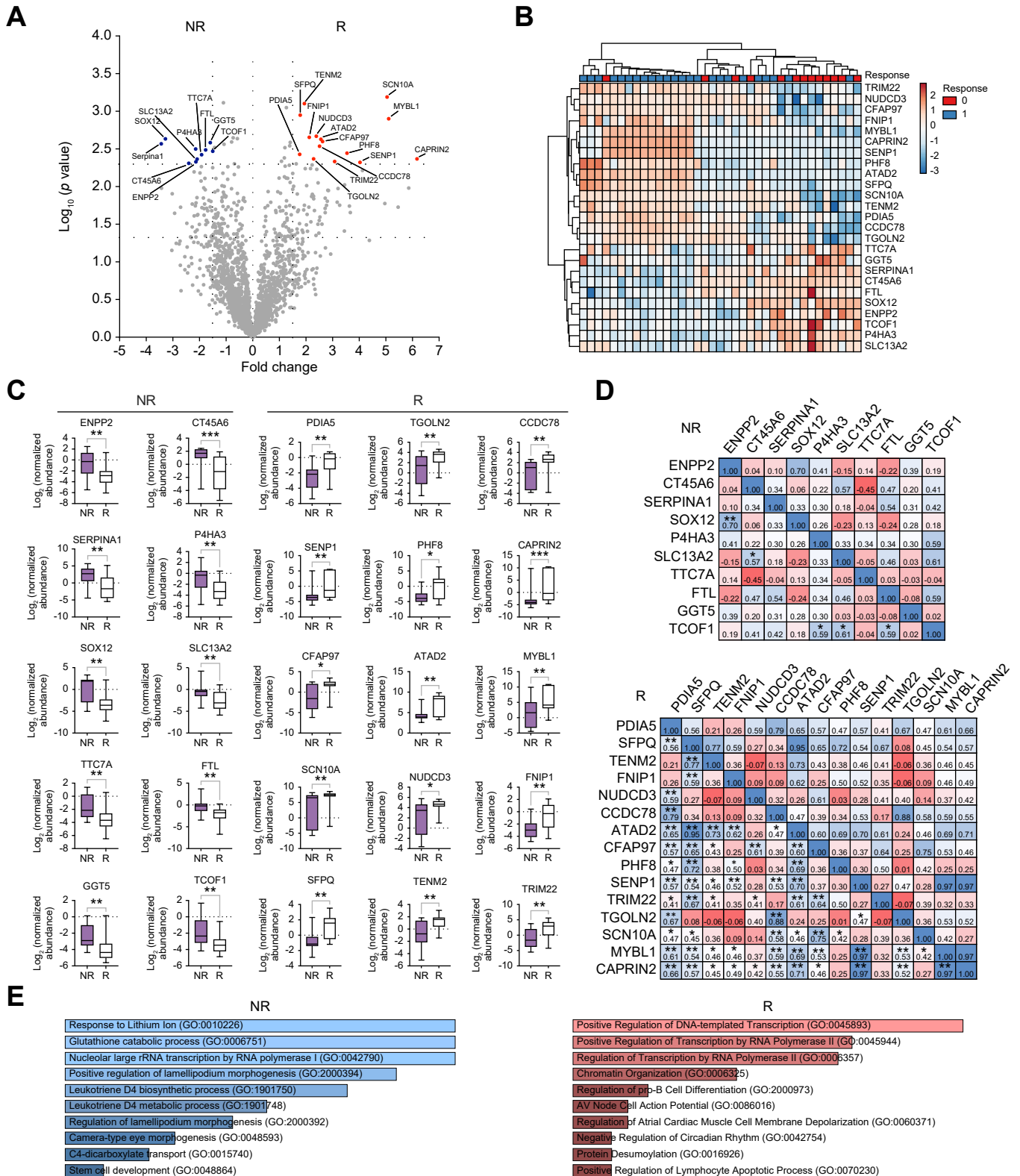


Fig. 2. Analysis of the differential protein abundance and association with survival risk. (A) Volcano plot showing EV proteins whose levels differentiate responders (R) from non-responders (NR) based on Student's t-test analysis. Red dots indicate proteins with higher levels in responders, while blue dots indicate proteins with higher levels in non-responders. (B) Heatmap of the differential level of 25 EV proteins. Legend: 1 = R, 2 = NR. (C) Protein expression levels were compared between responders (white boxes) and non-responders (black boxes) using the Mann-Whitney *U* test. Statistical significance is indicated by *p* values above each comparison. (D) Spearman correlation correlogram of proteins significantly associated with response. (E) Enrichment network for upregulated proteins in NR and R (**p* < 0.05, ***p* < 0.01). EV, extracellular vesicle.

Table 2. Survival analysis and predictive performance of EV-associated proteins. Statistically significant *p* values are indicated in bold.

	<i>p</i> value	AUC		<i>p</i> value	AUC
ATAD2	0.061	0.789	PHF8	0.061	0.753
CAPRIN2	0.035	0.798	SCN10A	0.0098	0.619
CCDC78	0.00028	0.783	SENP1	0.035	0.792
CFAP97	0.0098	0.679	SERPINA1	0.056	0.714
CT45A6	0.21	0.738	SFPQ	0.13	0.75
ENPP2	0.0094	0.658	SLC13A2	0.0077	0.625
FNIP1	0.29	0.777	SOX12	0.012	0.658
FTL	0.13	0.738	TCOF1	0.00011	0.705
GGT5	0.02	0.524	TENM22	0.84	0.622
MYBL1	0.2	0.69	TGOLN2	0.0019	0.708
NUDCD3	0.011	0.619	TRIM22	0.73	0.67
P4HA3	0.015	0.619	TTC7A	0.0059	0.616
PDIA5	0.0044	0.815			

The log-rank test was used to compare survival between groups, with significant differences indicated for each protein. To further evaluate the discriminative ability of the proteins for OS, ROC curve analysis was performed. The AUC is reported for each protein, with AUC >0.7 values indicating good predictive potential and AUC >0.8 strong predictive potential. EV, extracellular vesicle; OS, overall survival; ROC, receiving operating characteristic.

reporting HR values and bootstrap internal sampling validation is given in Table 1S.

Identification of prognostic factors for overall survival

To identify clinical and molecular factors associated with patients' outcomes, we performed univariate and multivariate analyses to evaluate their impact on OS. Univariate analysis revealed significant associations with OS for the number of treated lesions ($p = 0.024$), Child–Pugh score ($p = 0.004$), AFP ($p = 0.013$) and the presence of chronic hepatitis B ($p = 0.011$). In contrast, no significant association was observed for sex, age at treatment, lesion size, cirrhosis, radiation dose per liver volume (Gy), radiation time, and chronic hepatitis C and OS (all $p > 0.05$). Multivariate Cox regression analysis identified baseline EV-TCOF1 (HR 6.126, 95% CI 1.31–28.62, $p = 0.021$), Child–Pugh score (HR 4.881, 95% CI 1.58–15.03, $p = 0.006$), and AFP levels (HR 1, 95% CI 1.00–1.00, $p = 0.005$) as independent predictors of shorter OS (Table 3). The remaining five proteins did not retain independent significance in the multivariate model, suggesting their associations with OS were not independent of other clinical and molecular variables.

Validation of prognostic value identifies TCOF1 as robust survival marker

To further assess the clinical relevance of the identified proteins, we validated their prognostic value using the publicly available database The Cancer Genome Atlas–Genomic Data Commons (TCGA-GDC).²³ Among the candidates, only TCOF1 consistently demonstrated significant associations with patient survival which was overexpressed in tumor tissues, thereby confirming its prognostic potential (Fig. 4A–C). In contrast, the remaining proteins did not show consistent survival correlations across datasets, suggesting that their prognostic utility may be limited. To confirm the tumoral origin of EV-TCOF1, we then conducted an immunohistochemical analysis in healthy tissues (as control) and tumor samples collected from a representative subset of patients with high (patient IDs: E51, E78, and E48) and low (patient IDs: E101, E59, and E83) levels

of EV-TCOF1 in plasma (Fig. 4D). A higher level of EV-TCOF1 was associated with a strong nuclear expression of the protein, whereas a low level of EV-TCOF1 was associated with both cytoplasmic and nuclear expression (Fig. 4E). Healthy tissues resulted TCOF1 negative (Fig. 3S) of TCOF1 in tumoral tissue.

Discussion

In this study, we aimed to identify EV-associated protein biomarkers that predict patient outcome following HDR-BT for HCC. Our comprehensive proteomic analysis revealed distinct EV protein signatures that differentiate responders from non-responders, with EV-TCOF1 emerging as a robust prognostic marker in multivariate regression analysis. These findings are novel in the context of local ablation with HDR-BT and align with previous research highlighting the crucial role of EVs in cancer biology. EVs facilitate tumor progression and metastasis by mediating communication within the tumor microenvironment across multiple cancer types²⁴ and contribute to immune evasion by modulating antitumor immunity.²⁵ Although these studies primarily addressed cancers such as breast and lung, recent HCC-specific investigations have also underscored the importance of EV cargos in reflecting tumor aggressiveness and treatment resistance.¹¹ Our data corroborate this by showing that EV proteins, involved in cell adhesion, secretion, and extracellular matrix remodeling, correlate with survival outcomes in patients with HCC undergoing HDR-BT. Clinically, this is relevant as HDR-BT offers a targeted local therapy option for unresectable HCC, yet patient responses remain heterogeneous. Therefore, the identification of EV-associated prognostic markers provides promising tools for non-invasive stratification and personalized treatment planning. Our findings indicate distinct protein network patterns in responders vs. non-responders. In particular responders displayed multiple significant correlations among proteins involved in transcription and chromatin regulation (SOX12, MYBL1, PHF8, ATAD2, SFPQ) and signaling (ENPP2, TENM2, SENP1, CAPRIN2), suggesting a coordinated network supporting therapy response. Notably EV-TCOF1 emerged with strong significance as predictive marker. TCOF1 is a nuclear/nucleolar protein involved in ribosome biogenesis,²⁶ DNA damage response,²⁷ and radioresistance.²⁸ Recently, its dysregulation has been linked to cancer progression.²⁹ Elevated expression of TCOF1 is frequently observed in various tumor types compared with normal tissues, and high levels are associated with poor prognosis. Mechanistically, TCOF1 promotes tumorigenesis by enhancing cell cycle progression, cellular senescence, and rRNA production, and it can regulate oncogenic pathways such as KRAS activation and epithelial–mesenchymal transition.³⁰ Immunohistological analysis of tumoral tissues confirmed the nuclear staining of TCOF1 in patients with high levels of EV-TCOF1, whereas in patients with low levels of EV-TCOF1 the distribution of the protein in the tissue was mainly cytoplasmic. This may reflect altered subcellular trafficking or impaired export mechanisms associated with EV biogenesis. Our data indicate that patients with high plasma EV-TCOF1 (and predominantly nuclear localization) are less likely to respond to therapy. This association, which needs to be validated on a larger cohort of patients, suggests that high plasma EV-TCOF1 and nuclear retention of TCOF1 in tissue may mark tumors that are therapy resistant and have

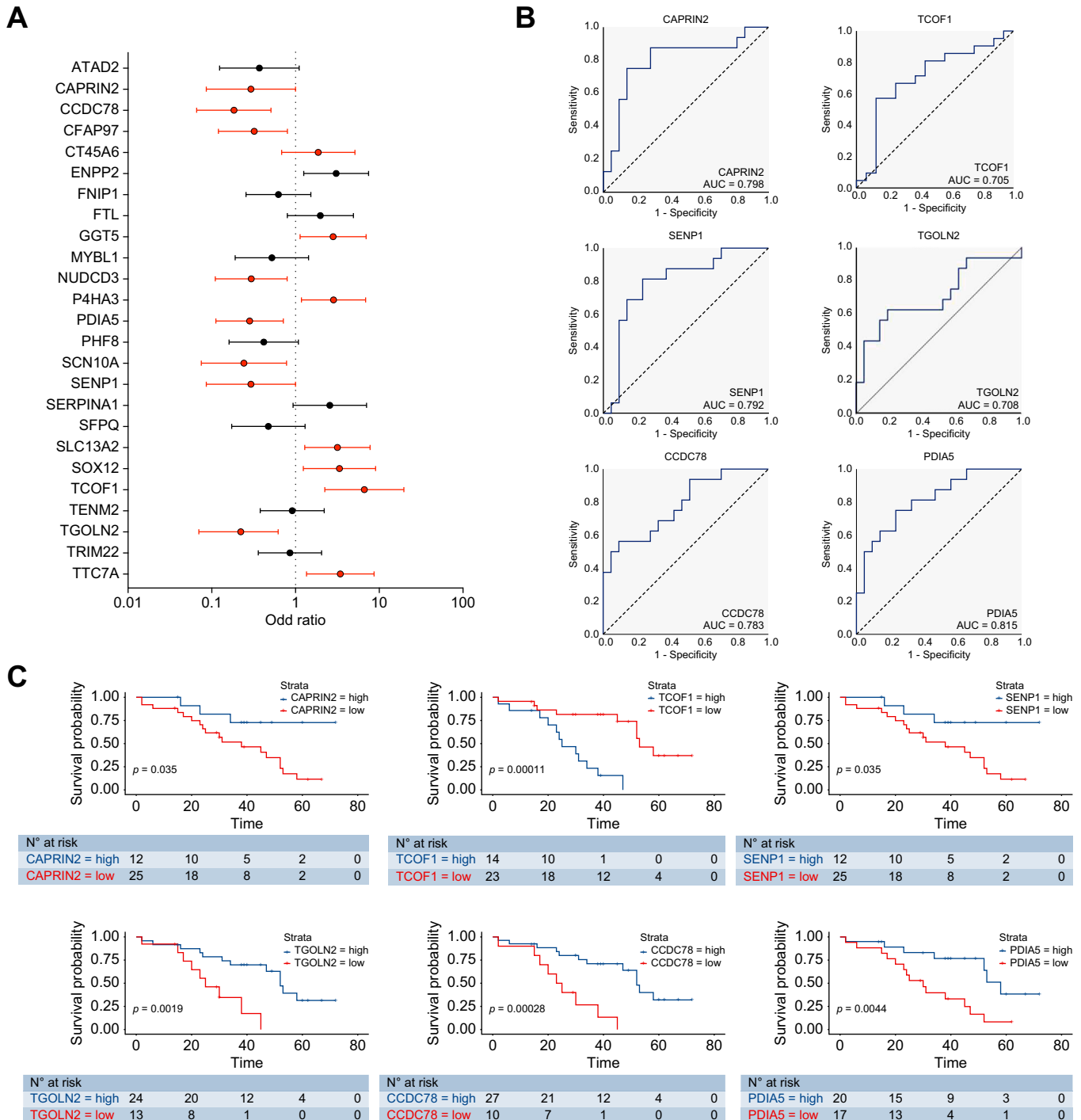


Fig. 3. Survival analysis and ROC curve assessment with AUC of EV-associated proteins. (A) Forest plot of the univariate Cox regression analysis showing hazard ratios and 95% confidence intervals for each variable. Significant associations ($p < 0.05$) are highlighted in red. Internal validation was performed by bootstrap Cox regression analysis based on 1,000 bootstrap samples. (B) ROC curves. The AUC is reported for each protein, with higher AUC values indicating stronger predictive potential. (C) Kaplan-Meier survival curves were generated for patients stratified by high and low expression levels of the identified EV proteins. The log-rank test was used to compare survival between groups, with significant differences indicated for each protein. The number of patients at risk at each time point is shown below the Kaplan-Meier plots. EV, extracellular vesicle; ROC, receiving operating characteristic.

active survival pathways, whereas low plasma EV-TCOF1 and cytoplasmic mislocalization may reflect altered intracellular trafficking and EV release. Importantly, although TCGA-based validation does not reflect a treatment-homogeneous HDR-BT cohort, it provides independent confirmation of tumor-intrinsic

prognostic relevance. Prospective validation in standardized radiotherapy cohorts will be required to establish EV-TCOF1 as a predictive biomarker in defined therapeutic settings.

Our group has previously reported data on EVs in patients from the SORAMIC study,³¹ undergoing locoregional therapy

Table 3. Univariate and multivariate Cox regression analyses of EV-associated protein and clinical predictors of OS. Statistically significant *p* values are indicated in bold.

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	<i>p</i> value	HR	95% CI	<i>p</i> value
TCOF1	6.642	2.24–19.69	<0.001	6.126	1.31–28.62	0.021
SENP1	0.293	0.08–0.99	0.05	0.823	0.20–3.35	0.786
PDIA5	0.283	0.11–0.71	0.008	1.778	0.27–11.54	0.547
CCDC78	0.188	0.06–0.52	0.001	0.904	0.55–1.47	0.691
TGOLN2	0.222	0.07–0.62	0.004	0.461	0.03–5.75	0.547
CAPRN2	0.293	0.06–0.69	0.049	1.560	0.78–3.10	0.205
Sex	0.725	0.26–2.02	0.538			
Age initial treatment	0.996	0.95–1.03	0.857			
Treated lesion	3.03	1.16–7.93	0.024	1.156	0.35–6.87	0.552
Lesion size	0.97	0.85–1.10	0.645			
Child–Pugh score (B vs. A)	3.923	1.55–9.89	0.004	10.262	1.99–52.86	0.006
Cirrhosis (yes vs. no)	3.74	0.49–28.32	0.201			
AFP	1	1.00–1.00	0.013	1	1.00–1.00	0.002
Gy/liver volume	0.999	0.99–1.00	0.094			
Time of radiation	1	0.99–1.00	0.951			
Chronic hepatitis B (yes vs. no)	4.451	1.42–14.49	0.011	1.24	0.19–7.73	0.817
Chronic hepatitis C (yes vs. no)	0.55	0.20–1.45	0.23			

EV, extracellular vesicle; OS, overall survival.

with radioembolization combined with systemic therapy with sorafenib or systemic therapy alone, demonstrating the significant prognostic and predictive potential of EV-GPX3, EV-ACTR3, and EV-ARHGAP1 for treatment response.¹¹ Notably, the EVs identified in that work differ from those found to be predictive and prognostic in HDR-BT in the present study. The observed differences between the EV-associated protein signatures identified in this study and those reported in the SORAMIC trial highlight the inherent challenges of biomarker discovery in radiation therapy for hepatocellular carcinoma. Variations in treatment modality (HDR-BT vs. external beam radiotherapy and vs. radioembolization), patient selection, disease stage, and analytical workflows may lead to context-dependent biomarker profiles. Rather than indicating contradictory findings, these discrepancies underscore the importance of interpreting biomarker signals within well-defined therapeutic and biological settings. Consequently, the present results should be regarded as hypothesis-generating and as a foundation for further functional and prospective validation.

The identification of EV-associated TCOF1 as an independent predictor of outcome following high-dose-rate brachytherapy raises the important question of how this biomarker could be translated into clinical practice. From a technical perspective, EV-based protein analysis is well suited for clinical implementation, as plasma sampling is minimally invasive and compatible with routine oncologic workflows. Standardization efforts would need to focus on robust and reproducible EV isolation (e.g. ultracentrifugation or size-exclusion chromatography) and targeted quantification of TCOF1, for example using antibody-based assays adaptable to a clinical laboratory setting.³² Clinically, EV-TCOF1 testing could be integrated into existing HDR-BT workflows as a pre-treatment risk stratification tool. Assessment of EV-TCOF1 levels before therapy may help identify patients at higher risk of non-response or early mortality, thereby informing treatment intensity, surveillance strategies, or consideration of alternative or combined treatment approaches.³³ Prospective validation in independent, multicenter cohorts will be a critical next step to define clinically relevant cut-offs and to evaluate the incremental value of EV-

TCOF1 beyond established clinical and imaging-based risk factors. Importantly, the translational potential of EV-TCOF1 extends beyond its role as a prognostic biomarker. Based on the strong association between elevated EV-TCOF1 levels and poor treatment response, TCOF1 emerges as plausible functional mediator of radioresistance in HCC. In this context, targeting TCOF1 may enhance radiosensitivity by impairing DNA damage repair and stress response pathways, and tumor-intrinsic modulation of TCOF1 may be reflected by corresponding changes in EV-associated TCOF1 levels. Establishing such a causal link would provide a biological rationale for future biomarker-driven therapeutic strategies combining radiotherapy with TCOF1-directed interventions. Together, these considerations position EV-TCOF1 as a promising candidate for clinical translation, with potential utility both as a minimally invasive prognostic biomarker and as a gateway to novel radiosensitization strategies in HCC.

Strengths of this study include prospective, standardized EV sampling within the ESTIMATE study and systemic monitoring of treatment response per protocol. A key limitation of this study is the absence of a formal dose–response analysis. All patients received a uniform HDR brachytherapy prescription of 15 Gy to the entire tumor volume (D100), including target margins, regardless of tumor size. As a result, dose–volume parameters, intratumoral dose heterogeneity, and biologically effective dose metrics could not be systematically analyzed in relation to EV-TCOF1 levels. Consequently, it cannot be definitively determined whether EV-TCOF1 exclusively reflects intrinsic tumor biology or partly captures interindividual differences in biological response to an identical delivered radiation dose. Future studies incorporating detailed dosimetric analyses, functional validation of TCOF1-mediated radiosensitivity, and prospective clinical evaluation will be required to resolve this question and to enable integration of EV-TCOF1 into dose-adaptive radiotherapy strategies. Additional limitations of the study are the relatively small sample size and the single-center design, which are limiting both the statistical power and the generalizability of the findings. Therefore univariate and multivariate analyses should be considered exploratory, and confirmation in larger, independent EV-based cohorts will be necessary. On this respect, the

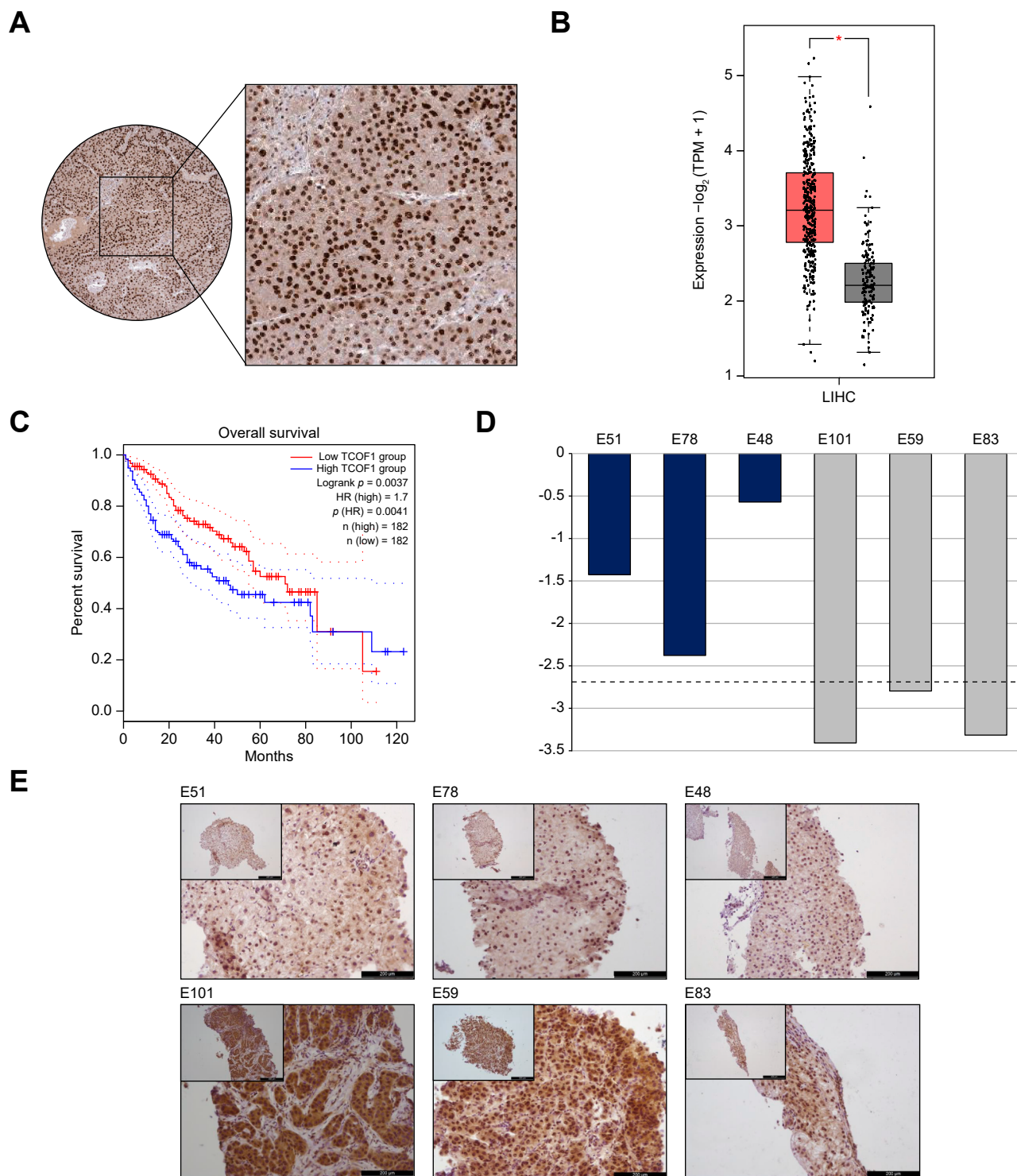


Fig. 4. Expression of TCOF1 in tumoral tissue. (A) Representative image from the Human Protein Atlas illustrating nuclear localization of TCOF1 in HCC tissue. (B) Box plot showing the differential expression of TCOF1 between tumor and normal tissues based on TCGA database. TCOF1 expression is significantly higher in tumor tissues ($p < 0.05$; $n = 369$, red box) compared with normal tissues ($n = 160$, gray box). Differential analysis was performed using the selected datasets ('TCGA tumors vs. TCGA normal + GTEx normal' or 'TCGA tumors vs. TCGA normal'). The method for differential analysis was one-way ANOVA, using disease state (Tumor or Normal) as the variable for calculating differential expression. (C) Kaplan–Meier analysis based on TCGA databases showing that patients with HCC ($n = 181$) with high TCOF1 expression have a significantly ($p = 0.014$) shorter OS with respect to patients ($n = 180$) with low TCOF1 expression. (D,E) Comparison of EV-TCOF1 levels in the plasma (D) with TCOF1 expression in tumor tissue (E) from representative patients. Magnification: $10 \times$ (scale bar: $400 \mu\text{m}$) and $20 \times$ (scale bar: $200 \mu\text{m}$). HCC, hepatocellular carcinoma; OS, overall survival; TCOF1, treacle ribosome biogenesis factor 1; TCGA, The Cancer Genome Atlas.

analysis of TCGA data was used to support the tumoral origin of EV-TCOF1 and the biological plausibility of its involvement in HCC but does not represent validation of circulating EV biomarkers. We must also recognize that HDR-BT, as a form of high-dose conformal radiotherapy, is currently available only at a limited number of specialized centers worldwide but consistently demonstrates excellent local control rates, a favorable safety profile, and an OS benefit for patients with HCC.³⁴ Its therapeutic outcomes appear broadly comparable to those reported for SBRT, which likewise delivers highly conformal, ablative radiation doses.^{34–36} Given the shared biological rationale of both modalities—precise, locally ablative high-dose treatment—it is plausible that insights gained from HDR-BT, including potential biomarkers or predictors of treatment

response, may also be relevant in the context of SBRT. However, direct prospective comparisons are needed to substantiate this assumption. In conclusion, the integration of EV biology for HCC therapy response prediction as a tool of liquid biopsy is innovative and in HDR-BT unique. Our data demonstrate that EV proteomics hold promise as prognostic and predictive biomarkers for patients with HCC undergoing local ablation with HDR-BT with curative intent. Particularly, EV-TCOF1 was found to hold excellent discriminative capacities in liquid biopsy, underlining the potential of EVs as non-invasive tools for response prediction and personalized treatment guidance in HCC. Integrating EV profiling into future clinical decision-making could significantly advance individualized management strategies in HCC.

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Abbreviations

AFP, alpha-fetoprotein; BCLC, Barcelona-Clinic Liver Cancer; DEP, differentially expressed protein; EV, extracellular vesicle; GDC, Genomic Data Commons; GO, Gene Ontology; HCC, hepatocellular carcinoma; HDR-BT, high-dose-rate interstitial brachytherapy; HR, hazard ratio; MRI, magnetic resonance imaging; MSN, Moesin; NR, non-responders; OS, overall survival; R, responders; ROC, receiving operating characteristic; SBRT, stereotactic body radiotherapy; TCGA, The Cancer Genome Atlas; TCOF1, treacle ribosome biogenesis factor 1; TRPS, tunable resistive pulse sensing.

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Conflicts of interest

NBK has received reimbursement of meeting attendance fees and travel expenses from EISAI and a lecture honorarium from Falk and Astra Zeneca. IP reports reimbursement of travel expenses and lecture honoraria from Roche and received lecture honoraria from Falk Foundation and third-party funding for scientific research from Novartis unrelated to this manuscript. All other authors have no competing interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Designed the study: DP-W, EÖ, MPF, MA-F. Curated the clinical data: DP-W, LS, MSt, NBK, IP, MPF. Performed the analysis: EÖ, HH-E, MA-F. Provided resources: SC, MW, MS, JR. Acquired funding: DP-W, MPF. Analyzed and interpreted the data: DP-W, EÖ, HH-E, MPF, MA-F. Supervised the project: PDP-W, MPF, MA-F. Wrote the original draft: EÖ, DP-W, MPF, MA-F. Read and reviewed the manuscript for important intellectual content: all authors. Approved the final manuscript: all authors.

Data availability

Data are available from the corresponding author on reasonable request.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2026.101853>.

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Author names in bold designate shared co-first authorship

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